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Effects of global warming on species with temperature-dependent sex determination: Bridging the gap between empirical research and management.

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Abstract

Global warming could threaten over 400 species with temperature-dependent sex determination (TSD) worldwide, including all species of sea turtle. During embryonic development, rising temperatures might lead to the over-production of one sex, and, in turn, could bias populations' sex ratios to an extent that threatens their persistence. If climate change predictions are correct, and biased sex ratios reduce population viability, species with TSD may go rapidly extinct unless adaptive mechanisms, whether behavioural, physiological or molecular, exist to buffer these temperature-driven effects. Here, we summarize the discovery of the TSD phenomenon and its still elusive evolutionary significance. We then review the molecular pathways underpinning TSD in model species, along with the hormonal mechanisms that interact with temperatures to determine an individual's sex. To illustrate evolutionary mechanisms that can affect sex determination, we focus on sea turtle biology, discussing both the adaptive potential of this threatened TSD taxon, and the risks associated with conservation mis-management.

Keywords: Climate change, temperature-dependent sex determination, reptiles, sex steroid hormones, sea turtles

Introduction

The current rate of species loss is often referred to as the sixth mass extinction event in geological history (Barnosky *et al.*, 2011). As climate change progresses and global temperatures continue to rise rapidly, understanding how species interact with their environment has become extremely important (Visser, 2008; Hoffmann *et al.*, 2011; Stocker *et al.*, 2013; Neukom *et al.*, 2019). This is particularly true for over 400 fish and reptile species that have temperature-dependent sex determination systems (TSD). For these species, incubation temperatures differentially trigger the pathways that lead to male and female gonad development (Charnov *et al.*, 1977; Deeming *et al.*, 1988). As global warming continues, the TSD mechanism could lead to heavily skewed offspring sex ratios towards one sex, which in turn threatens populations' persistence (Mitchell *et al.*, 2010; Laloë *et al.*, 2014; Eberhart-Phillips *et al.*, 2017). Whether TSD species will be able to withstand the rates of predicted temperature change and maintain viable sex ratios will depend on their adaptive potential (Eizaguirre *et al.*, 2014; Tomillo *et al.*, 2020). Adaptive potential is defined as "the ability of populations/species to respond to selection by means of phenotypic or molecular changes", and interacts with population structure and demography (Eizaguirre *et al.*, 2014; Rey *et al.*, 2020). Even though knowledge on the adaptive potential of TSD is essential for conservation management, it remains uncharacterised in most species, such as sea turtles (Tomillo *et al.*, 2020).

To understand pressures on sea turtles, it is essential to acknowledge that in addition to anthropogenic climate change, they face cumulative impacts from other human-induced stressors (McMahon and Hays, 2006; Hawkes *et al.*, 2007; Witt *et al.*, 2010), such as coastal development (Kaska *et al.*, 2013; Von Holle *et al.*, 2019), fisheries bycatch (Fossette *et al.*, 2014; Senko *et al.*, 2014) and illegal harvest of both eggs and adults (Tomillo *et al.*, 2008; Senko *et al.*, 2014). As a consequence, many populations are already depleted or in decline, and subject to extensive conservation management plans (Mortimer *et al.*, 2008; Hamann *et al.*, 2010; Wallace *et al.*, 2013). The actions to protect sea turtles from the effects of global warming are further limited by the inability to determine a neonate's sex non-lethally and the difficulty in justifying the sacrifice of individuals from endangered populations, both of which restrict TSD research in this taxon. As such, most interest in trying to quantify how sea turtles will adapt to climate change has focused on nesting behaviour such as phenological changes and site selection (Refsnider *et al.*, 2013; Mazaris *et al.*, 2013; Reneker and Kamal, 2016; Patrício *et al.*, 2017). Similarly, most approaches to mitigate the effects of rising temperatures have involved human manipulation of nest temperatures through, for example, relocation (either *in situ* or in hatcheries) and shading

(Mrosovsky, 2006; Tuttle *et al.*, 2010; DeGregorio *et al.*, 2011). Here, we review the current knowledge and highlight the importance of the adaptive potential of TSD mechanisms, by bridging empirical research gained from TSD model species with the more practical management of wild populations of sea turtles. We chose to highlight molecular and physiological responses, which we consider to be under-represented in sea turtle research in comparison to behavioural adjustments (Reneker *et al.*, 2016; Patrício *et al.*, 2017). Finally, we discuss how failing to consider the adaptive potential and underlying mechanisms of TSD in sea turtles could lead to inappropriate management decisions.

TSD patterns and environmental covariates

TSD species have no sex chromosomes. Instead, sex-determining genes are scattered across the genomes, and male- or female-determining pathways are triggered by temperature during a thermosensitive period of development (Charnov *et al.*, 1977; Bachtrog *et al.*, 2014; Shen *et al.*, 2014). This mode of sexual development was first reported in the common agama lizard, *Agama agama* in 1966 (Charnier, 1966). It has since been confirmed as the sex determining mechanism of several reptile lineages, including the tuatara, crocodilians and turtles (Janzen *et al.*, 1991; Cree *et al.*, 1995)

Different patterns of TSD exist (Figure 1). In Type Ia TSD, seen in most turtle species, males develop at cooler temperatures while females are produced under warmer conditions (e.g. the painted turtle, *Chrysemys picta*, Bull and Vogt, 1979). In Type Ib, this pattern is reversed, and males are produced at warm temperatures (e.g. the tuatara, *Sphenodon punctatus,* Cree *et al.*, 1995). Finally, species with Type II TSD, common to all crocodilians, produce males at intermediate temperatures and females at both hot and cold extremes (e.g. the American alligator, *Alligator mississippiensis,* Ferguson *et al.*, 1983; González *et al.*, 2019). Under constant incubation temperatures, the TSD thermal response curve is described by i) a pivotal temperature, at which an equal number of embryos within a clutch develop as males and females, and ii) the range of temperatures under which either male or female offspring may be produced, known as the transitional range of temperatures (Figure 1, Mrosovsky *et al.*, 1991; Girondot, 1999).

Genetic sex determination (GSD) and TSD are often considered to be mutually exclusive mechanisms, but instead should be regarded as two ends of a continuum, with environmental variation interacting with genetic mechanisms to different extents across species (Quinn *et al.*, 2007, 2011; Pen *et al.*, 2010; Bachtrog *et al.*, 2014; Holleley *et al.*, 2015, 2016). For instance, the

montane lizard, *Bassiana duperreyi*, has heteromorphic sex chromosomes, and eggs incubated at warm temperatures, characteristic of lowland environments, produce equal numbers of male and female offspring. Yet, when eggs are incubated at cool temperatures representative of high altitude summers, the ratio is skewed towards 70% male hatchlings, suggesting that temperature can partly override genetic triggers in this species (Shine *et al.*, 2002). In mammals, which have GSD, maternal condition can alter sex ratios (Trivers *et al.*, 1973; Sheldon *et al.*, 2004; Grant, 2007), moderated by the environmental conditions during the breeding and gestation periods (Edwards *et al.*, 2019).

Temperature is the primary determinant of gonad differentiation in TSD species, and the thermal environment of nests varies with substrate albedo (Hays *et al.*, 2001), shading (Refsnider *et al.*, 2013) and nest depth (Telemeco *et al.*, 2009; Tomillo *et al.*, 2017), which can all influence egg development. These variables differ depending on individual nest site selection and seasonality, and thus introduce variation into offspring sex ratios within and among populations (Reneker & Kamal, 2016). It is also known that precipitation and humidity interact with thermal conditions, and hence influence TSD. For instance, the relationship between rainfall and sex ratios has generally been attributed to the cooling effect of rain on the temperature of nesting substrate thanks to evaporative cooling (Godfrey *et al.*, 1996; Matsuzawa *et al.*, 2002; Houghton *et al.*, 2007; Lolavar *et al.*, 2015). An argument has recently also been made to suggest that humidity itself has an effect on sex ratios beyond that of temperature alone (Lolavar *et al.*, 2017, 2020). While these studies have not yet robustly demonstrated the effect of humidity on TSD, they do illustrate several points. Firstly, temperature does not act in isolation in natural environments. Secondly, we still do not fully understand all the factors that influence of sex determination in TSD species. As such, we should consider the potential for elements of the thermal response curve (e.g. the pivotal temperature and transitional range of temperatures) to vary from traditional values - either from plastic responses or as a result of adaptive evolution (Santidrián Tomillo *et al.*, 2020).

The Evolutionary Significance of TSD

A comprehensive theory explaining the selective advantage of TSD still seems to evade researchers (Sarre *et al.*, 2004; Pen *et al.*, 2010; Quinn *et al.*, 2011). While the random segregation of sex chromosomes in species with GSD reflects parents' equal chromosomal investment in male and female offspring, conforming to frequency-dependent selection, no such theory exists to easily explain the evolutionary significance of TSD (Fisher, 1930). Instead, three relatively robust hypotheses for the evolution of TSD have been suggested, with varying levels of support; i) the Charnov-Bull model of differential fitness, ii) the "Mighty Males" hypothesis, and iii)

the phylogenetic inertia hypothesis (Charnov *et al.*, 1977; Girondot *et al.*, 1999; Shine, 1999; Janzen *et al.*, 2006; Rollinson, 2019).

The Charnov-Bull model of differential fitness suggests that sex-specific advantages are associated with particular thermal environments, and that the TSD mechanism ensures the production of sexes at their optimal temperatures (Charnov *et al.*, 1977). The theory requires a heterogeneous environment, in time or space, where males and females benefit from different conditions (Charnov *et al.*, 1977). Sex-specific benefits from particular thermal environments emerge through different mechanisms. For instance incubation temperature may i) affect fitness proxies, such as growth rate or size, ii) correlate with future conditions that offspring will experience, or iii) affect developmental rates and timing of emergence (Shine, 1999; Janzen *et al.*, 2006). Recently it was suggested that sex-specific fitness may stem from bimodal age at maturity distributions, as TSD species show greater dimorphism in this trait than GSD species (Bókony *et al.*, 2019).

In TSD species the effects of temperature and sex overlap, and therefore it is challenging to disentangle their relative contributions to an individual's fitness (Rhen *et al.*, 2004). This problem has generally been overcome with the use of exogenous hormone manipulation experiments, whereby a given sex is artificially forced to develop at temperatures that would not otherwise enable its production. One of the best examples of such an experiment focused on the jacky dragon, *Amphibolurus muricatus* (Warner *et al.*, 2008). Eggs from this agamid lizard were incubated at a range of temperatures, and half of them were treated with an aromatase inhibitor that forced embryos to develop as male, regardless of their thermal environment. This manipulation decoupled the effects of sex and temperature on fitness and revealed that lifetime reproductive success was greater for males that were incubated at natural male-producing temperatures (Warner *et al.*, 2008). Importantly, hormone treatment had no effect on the morphology or survivorship of juvenile jacky dragons, indicating no effect of the treatment itself. Since this seminal study, further experiments have produced similar results across other agamid species (Steele *et al.*, 2020). While this reproducibility gives weight to the Charnov-Bull hypothesis, whether these results can be extrapolated to all TSD species, and particularly long-lived ones, remains unclear (Steele *et al.*, 2020). This is because there are conflicting results among experiments testing the Charnov-Bull model (Janzen *et al.*, 2006). For instance, studies using the diamondback terrapin *Malaclemys terrapin* failed to find support for the Charnov-Bull model (Morjan *et al.*, 2003). As such, caution is needed when presenting the Charnov-Bull model as a universal explanation for TSD in reptiles (Janzen *et al.*, 2006).

Recently, Rollinson (2019) proposed an alternative theory for the evolution of TSD, referred to as the "Mighty Males" hypothesis, based on the maternal condition hypothesis described by Trivers and Willard (1973). The original hypothesis posits that females' lifetime reproductive success is mostly constrained by the number of gametes they produce and, as such, even lower quality female offspring produced under sub-optimal conditions should not suffer a reduction in lifetime reproductive fitness. On the other hand, males' reproductive success is limited by their ability to compete for mating opportunities, and therefore male offspring should be produced under the environmental conditions that maximise their overall fitness (Trivers *et al.*, 1973). Given these constraints are related to sexual reproduction, independently of TSD, and given that temperature affects a wide range of traits such as size, developmental rate, or embryonic mortality, Rollinson (2019) proposes that males should be produced at the temperatures that maximise the fitness potential of these traits, while females should be produced at the sub-optimal extremes. As mortality increases with high temperatures, the Mighty Males hypothesis can be easily applied to type II (female-male-female) and type Ia (male-female) TSD species (Santidrián Tomillo *et al.*, 2020). On the other hand, it may not apply to type Ib (female-male) TSD species, unless evolutionary mechanisms exist that can significantly push back the upper temperature limits for mortality. Type Ib is rare in nature, existing in the tuatara and shorter-lived squamata groups, and may require an alternative explanation.

Finally, the difficulty in determining the evolutionary significance of TSD may stem from phylogenetic inertia and the possibility its adaptive significance may no longer be detectable 300 million years after it evolved (Janzen *et al.*, 2004, 2006). After phylogenetically reconstructing the evolution of sex determination in over 400 species of squamata, many examples of transitions from TSD to GSD were identified, but there were no cases where this direction was reversed (Pokorná *et al.*, 2009). It was therefore suggested that sex chromosomes may evolve when sex-specific genes are coupled with genes that provide a selective advantage to that sex. As the association between these genes strengthens and rates of recombination decrease, the conditions are met for sex chromosomes to evolve (Bachtrog *et al.*, 2014; Muralidhar *et al.*, 2018).

Heritability, Development and Molecular Pathways of Sex Determination.

Despite very different sex determination mechanisms across vertebrate taxa, similar genetic pathways underpin the molecular foundations of gonad differentiation. This is consistent with conserved patterns of gonad development across vertebrates, with gonads being initiated as a bipotential genital ridge in both GSD and TSD species. From a developmental perspective, the

genital ridge is formed from the coelomic epithelium, underlying mesenchymal cells and germ cells, which migrate into the ridge from the embryonic yolk sac (Morrish *et al.*, 2002). As the somatic and germ cells proliferate, the genital ridge expands until the cell fate commits to developing as testes or ovaries. The differentiation of testes necessitates the development of the primary sex cords into testis cords, whereas the formation of ovaries requires the thickening of the coelomic epithelium while the primary sex cords disappear (Morrish *et al.*, 2002). Importantly, depending on the species and the TSD pattern, the molecular mechanisms of the sex-determining period can either end before signs of gonad differentiation appear, or may overlap with the early stages of gonad differentiation (Morrish *et al.*, 2002).

Overall, there is no doubt about the role of temperature in sex determination of TSD species, yet individual responses, reflected in traits such as pivotal temperature, are underpinned by a genetic contribution with some levels of heritability, estimated in the painted turtle, *Chrysemys picta*, to be as high as h^2=0.35 (McGaugh *et al.*, 2011). It is therefore not surprising that studies have focused on elucidating the molecular mechanisms of sex determination (Martínez-Juárez *et al.*, 2019). Conventionally, genes are classified based on their GSD sex-specific pathways and probably all known mammalian sex determining genes have now been tested for their role in TSD (Rhen and Schroeder 2010). A very thorough gene-based overview can be found in Martínez-Juárez & Moreno-Mendoza (2019). In brief, the expression of genes such as *Foxl2* (Forkhead box protein L2) and *Rspo1* (encoding the protein R-spondin-1) becomes greater in the female determining pathway, but these are also detectable in the male cascade during early development (Elf 2003). Furthermore, *Dax1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region), *Sf1* (steroidogenic factor 1) and *Wnt4* (Wingless-Type MMTV Integration Site Family, Member 4), all involved in female development and the repression of male traits, also appear to contribute to the sex determination of TSD species (Shoemaker and Crews 2009). Conversely, in the male pathway, genes necessary for testicular differentiation include *Sox9* (SRY-Box Transcription Factor 9), the anti-Mullerian hormone (*AMH*) and *Dmrt1* (Doublesex And Mab-3 Related Transcription Factor 1). The expression of *Sox9*, a gene that belongs to the same HMG-box transcription factor as *Sry* (Sex determining region Y), is detectable in early gonad development and becomes restricted to the developing testis at the end of the thermosensitive period (reviewed in Rhen and Schroeder 2010). Noteworthy, the expression of *Sry* remains elusive in reptiles. *Dmrt1* is particularly interesting and shows temperature-dependent sex-specific expression that precedes gonadal sex differentiation. *Dmrt1* expression changes with shifts in temperature in a sex-specific manner, and also responds to the presence of aromatase inhibitors – regulation of this gene has been shown to be necessary in order to initiate male development in

Trachemys scripta (Ge *et al.*, 2017).

While the identification of these genes follows a candidate gene approach, in more recent years studies of TSD have focused on gene discovery. Along this vein, several new candidates have emerged. A single nucleotide polymorphism in *Cirbp* (Cold Inducible RNA Binding Protein) was associated with transcript levels in the embryonic gonads of the snapping turtle (*Chelydra serpentina*) during specification of gonad fate and hatchling sex (Schroeder *et al.*, 2016; Rhen *et al.*, 2017). The A allele was induced in embryos exposed to a female-producing temperature, while expression of the C allele did not differ between female- and male-producing temperatures. As such AA homozygotes were more likely to develop ovaries than the CC homozygotes which all developed as males, with the AC heterozygotes standing at an intermediate frequency. From an ecological perspective, it is worth noting that changes in allele frequencies in *Cirbp* were detected at small and large geographical scales, suggesting local adaptation. Such patterns of local adaptation would be expected to result in higher pivotal temperatures in warmer regions, possibly mediated by resistance thresholds to the development of the high temperature sex, linked to genetic underpinnings. On the other hand, in the central bearded dragon (*Pogona vitticeps*), a species in which chromosomal sex determination is overridden at high temperatures, sex-reversed females are produced when an intron is retained in the mature transcripts from each of two Jumonji family genes, *Jarid2* (Jumonji And AT-Rich Interaction Domain Containing 2) and *Jmjd3* (Jumonji Domain Containing 3, Histone Lysine Demethylase). This intron retention was observed only in females that have been sex-reversed by temperature, not in classic chromosomal females or males (Deveson *et al.*, 2017). Similarly to *Cirbp* in *C. serpentina*, if the central bearded dragon has evolved mechanisms to regulate the over production of a given sex, we should be able to observe spatial and temporal variation of this intron loss in relation to temperature clines.

De-novo genome sequencing has also introduced elements of gene and mechanism discovery. This has been the case with the assembly of American alligator genomes, which, combined with RNA sequencing and models of CTCF-mediated chromatin looping, identified genomic regions that were significantly enriched for genes with female-biased expression in developing gonads after the thermo-sensitive period (Rice *et al.*, 2017). This approach demonstrated that estrogen signalling is a major driver of female-biased gene expression and holds promises for comparative genomics of TSD species.

Finally, a common pattern that has emerged from genomic studies is that the discovery of new TSD associated genes does not appear to be independent of epigenetic mechanisms. For

instance, the DNA methylation dynamics of the *Dmrt1* promoter region are tightly correlated with temperature and could mediate the impact of temperature on sex determination (Ge *et al.*, 2018). In *T. scripta*, Ge et al (2018) demonstrated how the epigenetic regulator *Kdm6b* demethylates the histone H3 lysine 27 (*H3K27*) at the *Dmrt1* promoter region, in a process that results in male sex determination. It appeared though that *Kdm6b* is not in itself responsive to temperature, and as such the fundamental thermal trigger of this pathway remains unknown. Similarly, *Jarid2* is a component of the master chromatin modifier Polycomb Repressive Complex 2, and the mammalian sex-determining factor *Sry* is directly regulated by an independent but closely related Jumonji family member (Devison et al 2017). The authors proposed that the alteration of *Jarid2*/*Jmjd3* function by intron retention alters the epigenetic landscape to override chromosomal sex-determining cues, triggering sex reversal at extreme temperatures (Devison et al 2017).

It is difficult to predict the nature of selection exerted by global warming, as directional selection would be expected under a constant increase in temperature, but a predicted increased frequency of extreme events should result in fluctuating selection. When selection is constrained, then phenotypic plasticity may allow species to respond to their environment (Chevin *et al.*, 2010). Indeed, while epigenetics, as mechanisms of phenotypic plasticity, may appear distant to practical conservation, their relevance has recently been framed in the light of management decisions (Rey *et al.*, 2020). Altogether, understanding the mechanisms underlying phenotypic plasticity is a valuable direction of research in conservation management. In particular, here we suggest that increased consideration of endocrinology and sex steroid hormones would benefit the conservation of TSD species.

Plasticity and sex steroid hormones in TSD species

Physiological plasticity includes a series of mechanisms by which organisms can match their phenotypes to their environmental conditions, for instance via the endocrine system and hormone regulation (Gienapp *et al.*, 2008; Chevin *et al.*, 2010; Merilä *et al.*, 2014). In the specific context of phenotype-environment matching, evolutionary theory suggests that the populations demonstrating the greatest levels of plasticity in beneficial traits might have the highest potential of persisting in the face of climate change (Meyers *et al.*, 2002). This is because if the cost of plasticity is low, it will reduce the range of conditions under which extinction is inevitable (Chevin *et al.*, 2010; Sanford *et al.*, 2011). Plasticity could thus maintain populations until adaptive evolution emerges and improves the phenotype-environment match (Lande, 2009; Reusch, 2014).

Sex steroid hormones, such as estrogens and their precursors, androgens, interact with the molecular pathways that control gonad differentiation, and are fundamental for regulating gonad development across a wide variety of taxa (spanning amphibian (Ko *et al.*, 2008), bird (Nakabayashi *et al.*, 1998), fish (Wang *et al.*, 2007), mammals (Uhlenhaut *et al.*, 2009) and reptiles (Barske *et al.*, 2010)). Estrogens repress *Sox9* expression, preventing the differentiation of male gonad-specific Sertoli cells in both GSD (mouse, Uhlenhaut et al. 2009) and TSD species (*T. scripta,* Barske and Capel, 2010). Conversely, the development of the müllerian ducts, which provide the structure for female gonads, correlates with changes in estrogens in mammals, birds and reptiles (Dodd *et al.*, 2008). In TSD species, the estrogen 17β-estradiol replicates the effect of temperature in instigating demethylation of histone H3 lysine 27 (*H3K27*) at the *Dmrt1* promoter region, resulting in female gonad development (Ge *et al.*, 2018). Moderating embryonic exposure to sex steroid hormones is thus likely to be an effective mechanism for conferring plasticity to the TSD thermal response curve (Bowden *et al.*, 2000; Carter *et al.*, 2017).

The influence of androgens and estrogens on embryonic sex independently of temperature in TSD species has been extensively reviewed (Elf, 2003; Bowden and Paitz, 2018). To date, research exploring the relationship between steroid hormones and TSD pathways has largely focused on estradiol, testosterone and the enzyme aromatase (Box 1). Studies began with *in vitro* manipulation experiments which showed that exogenous application of estradiol produced female offspring at male-producing temperatures (Crews *et al.*, 1989, 1991; Wibbels *et al.*, 1991). At the same time, the application of aromatase inhibitors such as fadrozole, which prevent the synthesis of estradiol, have repeatedly resulted in male offspring (Wibbels *et al.*, 1994; Rhen *et al.*, 1995; Warner *et al.*, 2017). Patterns of gonad development in response to exogenous hormone application have not, however, always been predictable and negative results have been reported, along with incidences where exogenous application of estradiol unexpectedly produced male offspring (Janes *et al.*, 2007; Warner *et al.*, 2014).

The apparent inconsistencies that are reported both between *in vitro* experiments and across species can be partially explained by interactions between exogenous hormones and temperature. Estradiol and temperature produced more female embryos than would be expected by each treatment alone near the pivotal temperature of the red-eared slider turtle *T. scripta* (Wibbels *et al.*, 1991). Under extreme natural incubation temperatures (>2 °C above the 75 year nesting site average), exogenous application of estradiol and fadrozole had no effect on painted turtle sex ratios (Warner *et al.*, 2017). Yet, under average seasonal conditions exogenous estradiol produced more female offspring, and fadrozole more male offspring, than controls

(Warner *et al.*, 2017). These results suggest that temperature and hormones may have a dose-dependent and interactive effect on sex determination pathways (Wibbels *et al.*, 1991).

In oviparous species, a principal conduit of hormone transfer from mother to offspring is the egg yolk (Schwabl, 1993; Radder, 2007). It provides material with which to prime the reactions associated with the molecular cascades that trigger gonad differentiation. In European sea bass *Dicentrarchus labrax*, exposure to male producing temperatures results in methylation of the *Cyp19a1* gene and lower aromatase expression (Navarro-Martín *et al.*, 2011). Similar methylation patterns are also seen in red-eared slider turtles *T. scripta* (Matsumoto *et al.*, 2016). High levels of aromatase expression will increase the biosynthesis of estradiol from testosterone, if this substrate is available and the reaction is not inhibited (Boon *et al.*, 2012). Interestingly, the estradiol:testosterone ratio within egg yolks at oviposition (i.e. before synthesis has occurred) varies across reptile species, having been recorded as both above and below 1, but rarely spanning an equal ratio (Radder, 2007), suggesting species-specific regulation. Depending on species, endogenous estradiol in yolks could act on female-producing pathways directly, or indirectly through the aromatase synthesis of endogenous testosterone, to produce female hatchlings (Figure 2). However, in loggerhead sea turtles, the ratio of estradiol:testosterone in egg yolks can favour either hormone, or be equal (Lockley *et al.* 2020). In a natural experiment where temperatures were controlled among nests, equal, low concentration, estradiol:testosterone ratios produced male offspring, which the authors theorise is due to product-feedback inhibition of aromatase (Figure 2, Lockley *et al.* 2020). Such reactions will be occurring within the yolk of eggs used for exogenous application studies. The interference with natural estradiol:testosterone ratios that could occur in these experiments might thus explain those occasions where exogenous estradiol application has produced male offspring (Janes *et al.*, 2007; Warner *et al.*, 2014).

A fundamental constraint of our knowledge generated by exogenous application studies is therefore that the biological relevance of results within natural systems is extremely limited (Bowden *et al.*, 2018). Field studies are invaluable for elucidating how this mechanism interacts with environment. When clutches of painted turtles were incubated at constant temperatures of 28 °C, the endogenous estradiol:testosterone ratio within egg yolks correlated with a seasonal shift in sex ratio from 72% male to 76% female (Bowden *et al.*, 2002). This relationship resulted in thermal reaction curves systematically changing across a nesting season, in a manner that allowed sex-specific phenotypic matching, enhancing the production of female offspring under warm conditions (Carter *et al.*, 2017). Such a mechanism fits directly with the Charnov-Bull

differential fitness hypothesis, allowing nesting females to maximise female offspring production under temperatures that could be most beneficial to them. In addition, it is consistent with the "Mighty Males" hypothesis, favouring the production of female offspring under warm conditions that may increase mortality rates. This mechanism, however, could accelerate the rate of female production as temperatures rise and may therefore represent an example of evolutionary suicide (Bowden *et al.*, 2000).

Temperature-dependent Sex Determination and Sea Turtles

We have considered the many molecular pathways that contribute to the formation of gonads in TSD species, and outlined how sex steroid hormones can interact with temperature (Figure 3). We now focus on applying this gained knowledge to sea turtles and their management, in the light of global warming. Sea turtles are Type Ia TSD species (Yntema *et al.*, 1982) and thus thermal projections across the coming century suggest that offspring sex ratios will become increasingly feminised (Yntema & Mrosovsky 1982; Hawkes et al. 2007; Hawkes et al. 2009; Witt et al. 2010; Laloë et al. 2014; Tanner et al. 2019). These projections, however, do not generally account for the adaptive potential of populations (Santidrián Tomillo *et al.*, 2020).

Sea turtles demonstrate a high degree of natal philopatry to their nesting sites, often with high female fidelity and male-mediated gene flow among mating sites (Meylan *et al.*, 1990; Bowen *et al.*, 2007; Lee *et al.*, 2007; Stiebens *et al.*, 2013; Levasseur *et al.*, 2020). This strong female philopatry creates genetic structure within a rookery that is prone to the evolution of local adaptation (Stiebens *et al.*, 2013; Baltazar-Soares *et al.*, 2020). Female turtles return to nest at intervals ranging from 1 to 4 years, while males return more frequently, and can even remain resident to the mating grounds (Hays *et al.*, 2010; Schofield *et al.*, 2010; Arendt *et al.*, 2012). Different re-migration intervals between males and females result in different operational sex ratios (the ratio of sexually active males to females at the nesting site a given time) than that of adult sex ratios, which are likely to be less female biased than offspring sex ratios (Hays *et al.*, 2014). While the operational sex ratio and even mating strategies (e.g. Lee *et al.*, 2004) may alleviate offspring sex ratio bias, a reduction in the total number of males can have implications for populations: it can lead to reduced genetic diversity (Frankham, 2005), increase potential for inbreeding and genetic drift (Hedrick *et al.*, 2000), and ultimately reduce a population's fitness and its adaptive potential (Reed *et al.*, 2003).

TSD in Sea Turtles: The barriers

Little is known about TSD in sea turtles as, historically, sexing individuals has required sacrifice and histological examination of gonads (e.g. Wyneken *et al.*, 2007; Hamann *et al.*, 2010; Fuentes *et al.*, 2017). As most sea turtle populations are listed on the IUCN red list, the sacrifice of hatchlings is often restricted by local authorities. Over the years, several protocols have been developed to overcome this challenge, such as quantifying the estradiol:testosterone ratio in hatchling plasma (green sea turtle: 96.7% overall accuracy, n = 30, Xia *et al.*, 2011, loggerhead sea turtle: 94% accuracy for males and 100% accuracy for females, n = 28, Gross *et al.*, 1995). Along the same approach, the expression of Anti-Müllerian Hormone (*AMH*) seems to also effectively identify the sex of loggerhead sea turtle hatchlings, being expressed only in males (n = 59, Tezak *et al.*, 2020). These protocols will now need to be up-scaled to reach a high throughput, to lead a new era of research in the study of TSD and management of sea turtles.

Until large scale sexing techniques are possible, our overall knowledge of TSD thermal response curves in sea turtles is limited to laboratory incubation studies with small sample sizes (Rimblot *et al.*, 1985; Mrosovsky, 1988; Mrosovsky *et al.*, 1992; Godfrey *et al.*, 1999). These studies mostly identified the pivotal temperature of sea turtle populations to lie around 29 °C (but see McCoy *et al.* 1983; Wibbels, *et al.*, 1998). In more recent years, sophisticated modelling approaches have been used to refine the characteristics of thermal response curves (Abreu-Grobois *et al.*, 2020), and have also quantified the variation in this trait among populations (Bentley *et al.*, 2020). However, prior to these advances, 29 °C was often used as an approximate pivotal temperature to estimate sex ratios of sea turtle populations in cases where sacrificing hatchlings was not possible, using mean nest temperature during the middle third of incubation as a proxy (see Table 1 for illustrative cases). Alternatively, studies have used the thermal response curves described specifically for the population of interest or a nearby neighbour, if these values have been reported (Table 1).

The approach of using a pivotal temperature proxy during the middle third of incubation is now widely regarded to be too oversimplified, as the thermosensitive period is dependent on development rates, which are not linearly related to temperature, particularly under variable thermal environments (Georges *et al.*, 2005; Massey *et al.*, 2019). Importantly, it also does not account for individual variation in, or the adaptive potential of the pivotal temperature. Other measures that correlate with sex ratio, such as incubation duration, have also been used, but estimates tend to vary dependent on the proxy used (Fuentes *et al.*, 2017). For instance, Fuentes *et al.* (2017) demonstrated that estimating the sex ratio of natural nests using the constant temperature equivalent (which converts natural fluctuating incubation temperatures into a

constant value to be compared to temperatures produced under lab conditions (Georges *et al.*, 2005)) during the middle third of incubation, and assuming a pivotal temperature of 29.12 °C, predicted an average sex ratio of 5.99% (95% CI: <0.1% - 37%) male. This estimate increased to 9.97% (95% CI: <0.1% - 86.20%) male when using incubation duration as a proxy for the same nests. While the use of proxies has contributed to our general understanding of sex ratio distributions, their reliance on pre-existing thermal response curves make them unable to detect real-time deviation from such curves that might originate from different elements of the adaptive potential of sea turtles – conceivably with major consequences of past and future management decisions.

TSD in Sea Turtles: Adaptive Potential

To date, attention on the impact of global warming on the sex ratios of sea turtles has largely focused on how plastic behaviours can avoid extreme sex ratio biases. Such behaviours include nest site selection (Reneker & Kamal, 2016; Patrício *et al.*, 2017), nest depth (Refsnider *et al.*, 2013), and phenological shifts (Mazaris *et al.*, 2013). For instance, with rising temperatures there is evidence that nesting seasons start earlier and can be more protracted for loggerhead turtles nesting in North Carolina and Greece (Weishampel *et al.*, 2004; Hawkes *et al.*, 2007; Mazaris *et al.*, 2013; Patel *et al.*, 2016). From a global perspective, there is a significant negative relationship between the dates of first nesting for populations of loggerhead sea turtles across their nesting distribution, and the sea surface temperature at the beginning of the nesting season (Mazaris *et al.*, 2013). Despite evidence of phenological variation, the sole extent of such shifts is unlikely to be sufficient to keep up with the rate of contemporary climate change (Telemeco *et al.*, 2013; Monsinjon *et al.*, 2019).

Consequently, mechanisms other than behavioural adjustments may prove vitally important for sea turtles to respond to climate change. Local adaptation, which, in the case of TSD, would involve populations adjusting thermal response curves to match the changing thermal environment of their nesting sites, could be a fundamental part of this process. Patterns of local adaptation have been shown to evolve from the philopatric nature of sea turtles, as previously demonstrated for immune genes (Stiebens et al 2013) and even feeding strategies (Cameron et al 2019). For instance, green sea turtle hatchlings from dark sand (high temperature) beaches on Ascension Island grow faster and have higher levels of hatching success than those from nearby white sand (cool) beaches when exposed to hot artificial incubation environments (Weber *et al.*, 2012). Interestingly, no fine-scale adaptation of the pivotal temperature was found between turtles nesting on these two beaches in a later study, showing the context-dependence of the

results (Tilley *et al.*, 2019). On the other hand, flatback turtle *Natator depressus* eggs at tropical latitudes in Australia have high levels of tolerance to prolonged warming exposure up to 35 °C during incubation, despite these temperatures often being lethal (Maulany *et al.*, 2012; Howard *et al.*, 2015). The pivotal temperatures of three genetically distinct flatback turtle populations also vary by 1.5 °C, being highest in the population nesting under the warmest conditions (Bentley *et al.*, 2020). These records of adaptation to local thermal environments provide evidence that the temperature sensitive triggers of the molecular pathways controlling sex determination can evolve (e.g. Ewert *et al.*, 2005).

There is very little information on whether maternally derived sex steroid hormones can contribute to local adaptation, or whether they can introduce plasticity to the sea turtle TSD mechanism. This lack of attention to the evolvability of the endocrine system is unwarranted given the evidence from other TSD species (Bowden *et al.*, 2000; Ewert *et al.*, 2005). Experimental estradiol treatment of olive ridley turtle eggs at male producing temperatures can feminize gonads (Merchant-Larios *et al.*, 1997), disrupt testis differentiation (Díaz-Hernández *et al.*, 2015), reduce cell proliferation (Díaz-Hernández *et al.*, 2017), delay *Sox9* inhibition, and delay the upregulation of *FoxL2* and aromatase (Díaz-Hernández *et al.*, 2015). Furthermore, emerging research from a natural, *in situ* experiment that controlled for temperature found that the ratio of maternally derived estradiol:testosterone in the yolk of loggerhead sea turtle eggs correlated with the sex ratio of nests independently of temperature (Lockley *et al.*, 2020). Research that aims to evaluate the elements of the TSD mechanism that can evolve and locally adapt will be fundamental for revealing the adaptive potential of the TSD mechanism in sea turtles.

Implications for Conservation Management

Global efforts dedicated to the conservation of sea turtles are extensive, with hundreds of grassroot, national and international projects working to stop the decline of populations and promote their persistence. Such work frequently focuses on the protection of nesting beaches (Hamann *et al.*, 2010; Fuentes *et al.*, 2012). Along with the benefits of accessibility, logistical advantages and low cost options, protection at this point in the life cycle can reduce threats from, for example, poaching of eggs and adult turtles (Tomillo *et al.*, 2008; Senko *et al.*, 2014), tidal inundation (Varela *et al.*, 2019) and coastal development (Kaska *et al.*, 2013; Von Holle *et al.*, 2019).

A common conservation approach to mitigate the effects of human-induced stressors such as coastal development and poaching is to relocate egg clutches into *in situ* hatcheries, where nests

are protected and monitored (Mrosovsky, 2006, 2008; Pike, 2008; Pfaller *et al.*, 2009; Tuttle *et al.*, 2010). However, these relocations should not be performed lightly, as shading, substrate properties and depth can be different from the nesting beach, and can all substantially alter the local thermal regimes (Morreale *et al.*, 1982; Tuttle *et al.*, 2010; DeGregorio *et al.*, 2011). The differences between temperatures in hatcheries and *in situ* sea turtle nests vary between locations. For instance, no difference in temperature was recorded during the thermosensitive period between relocated and *in situ* loggerhead sea turtle nests in North Carolina, but over the entire incubation period nests in the hatchery were exposed to higher overall temperatures, and hatchlings from these nests emerged sooner (DeGregorio *et al.* 2011). However, this was not observed in Georgia, where there were no significant differences in temperatures, size or incubation duration between relocated and *in situ* nests, but there was reduced survival in the nests that had been relocated (Tuttle *et al.,* 2010). If nest relocation is undertaken, then continuous evaluation of incubation temperatures, durations and the effects on hatchling development and fitness should always be conducted.

Crucially, if done incorrectly, relocation may interfere with natural selection for traits that will help sea turtles adjust to new temperature regimes, or reduce the effectiveness of natural buffering mechanisms, as climate change progresses (Mrosovsky, 2006; Pfaller *et al.*, 2009). This effect may be particularly strong for these highly philopatric species, as they are the ones predicted to have evolved the strongest signature of local adaptation (Stiebens *et al.*, 2013; Baltazar-Soares *et al.*, 2020). Specifically, human manipulation of nest temperatures might dampen the effects of natural selection on developing embryos, by reducing selection on the genomic mechanisms that confer thermal tolerance and a higher pivotal temperature. Alternatively, if plastic phenotype-environment matching were occurring, either in the form of maternal hormone transfer or nesting behaviour, intentionally modifying incubation conditions would cause a human-induced thermal mismatch between the clutch and its optimal conditions.

It is thus important that if nest relocation is necessary to protect from stressors such as inundation or predation, this is done in a manner that matches the thermal environment that embryos would have experienced had eggs remained *in situ*. This includes taking all possible care to relocate nests to similar substrates in the vicinity of the nesting beach, in a nest cavity of the same depth and shape as originally dug by the female, with similar hydric properties. By doing this, conservationists will ensure that any behaviour is accounted for, while ensuring that unseen physiological plasticity such as hormone transfer will act in the direction of natural selection. In addition, in light of our current lack of knowledge on accurate sex ratios and adaptive

potential, relocating nests with the direct intention of manipulating temperature regimes (in an attempt to mitigate against global warming itself) risks preventing populations from adapting to conditions naturally. This leaves conservationists in a position where leaving egg clutches exposed to increasing temperatures is not viable, but altering incubation conditions by reducing temperatures may also have long-term negative consequences, whereby local adaptation is prevented or reduced. For scientists interested in TSD, focusing on developing non-lethal sexing methods that can be applied at a large scale would be extremely useful to guide such future conservation decisions.

Conclusions

Sea turtle conservation managers and scientists face difficult management choices as global warming progresses, and tools are still missing to make fully informed decisions about mitigation strategies. Barriers to determining neonate sex have limited our understanding of primary sex ratios in this taxon, and this knowledge gap must rapidly be overcome. It is likely sea turtles have evolved heritable mechanisms, whether behavioural, genetic, epigenetic or physiological, to respond to climate change, but these are not currently quantified.

To inform future decisions, we must accept it is not sufficient to estimate sex ratios based on theoretical pivotal temperatures from distant populations. We must begin to monitor sex ratios in a high throughput manner to quantify the adaptive potential of these species. This will enable us to assess the thermal response curves of populations and nesting aggregations, along with their variation across time and space. How this variation is maintained is an important question in the light of climate change and the underlying mechanisms will need to be clarified.

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Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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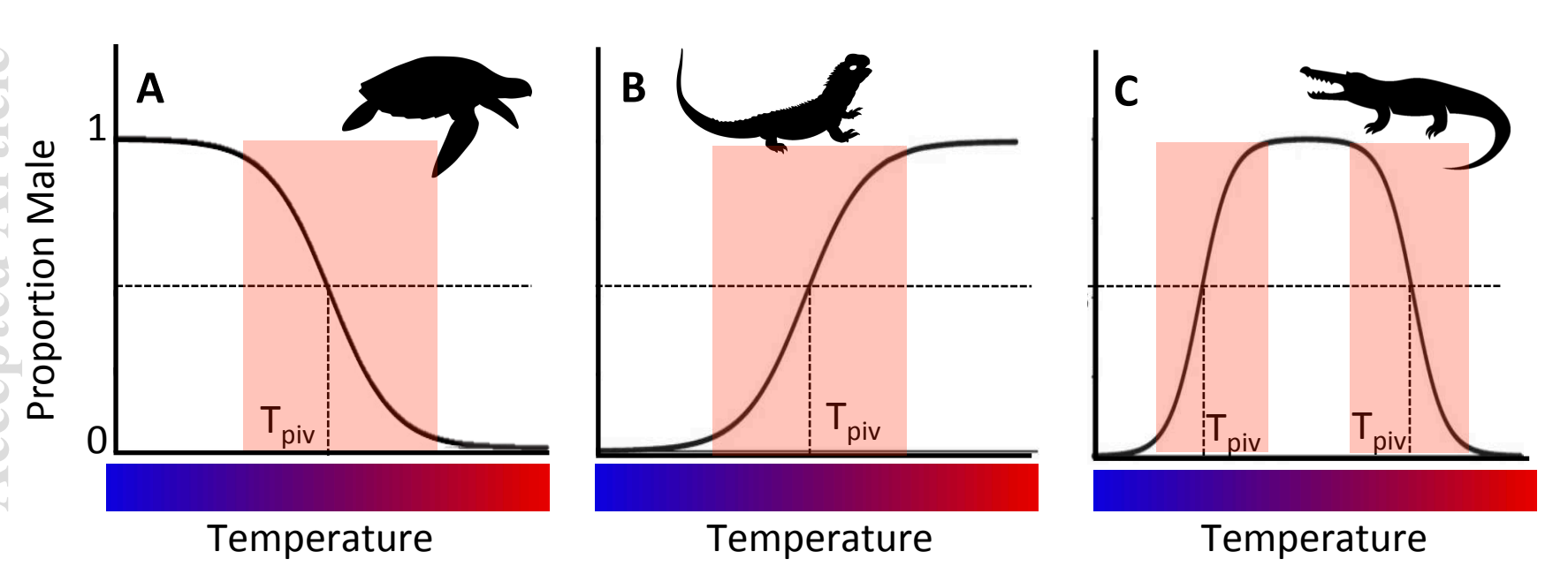
Box 1: An overview of Aromatase

Aromatase is an enzyme encoded by the *Cyp19a1* gene (Strauss *et al.*, 2018), which is part of the cytochrome P450 superfamily. This protein is the only known enzyme to catalyse the conversion of androgens to estrogens, a process that occurs throughout all vertebrate taxa. The P450 superfamily is an ancient lineage of genes that diverged early in the evolution of vertebrates (Simpson, 2004; Boon *et al.*, 2012; Nelson *et al.*, 2013), with gonadal synthesis of estrogens originating 500 mya (Lange *et al.*, 2002). *Cyp19a1* is highly conserved (Conley *et al.*, 2001). In fish and reptiles, there are two aromatase isomorphs encoded by the *Cyp19a1* and *Cyp19b1* genes and expressed in the gonads and brain respectively (Boon *et al.*, 2012). For most species, the majority of estrogen biosynthesis occurs in the gonads, with biosynthesis in the brain associated with behaviour (Simpson, 2004). In comparison, a single gene encodes human aromatase, with tissue specific promoter regions found on exon 1 of this gene, enabling its biosynthesis to occur in a greater range of tissues (Sebastian *et al.*, 2001; Bulun *et al.*, 2004). The up-regulation of *Cyp19a1* in the gonads is required for ovarian differentiation in fish (Guiguen *et al.*, 2010), birds (Smith *et al.*, 1997) and reptiles (Jeyasuria *et al.*, 1998), but not in mammals, where the knockout of *Cyp19a1* does not prevent ovaries from developing (Fisher *et al.*, 1998).

Species	Location	T_{piv} (°C)	Source of T_{piv}
Green (Esteban *et al.*, 2016)	Chagos Archipelago	29	Review (Ackerman, 1997)
Green (Broderick *et al.*, 2001)	Ascension Island	29	Review (Ackerman, 1997)
Green (Booth *et al.*, 2006)	Heron Island, Australia	27.5	T_{piv} previously calculated for population (Booth *et al.*, 2001)
Hawksbill (Esteban *et al.*, 2016)	Chagos Archipelago	29	Review (Ackerman, 1997)
Hawksbill (Glen *et al.*, 2004)	Antigua	29.2	Review (Mrosovsky *et al.*, 1991)
Leatherback (Santidrián Tomillo *et al.*, 2014)	Costa Rica	29.4	T_{piv} previously calculated for same population (Binckley *et al.*, 1998)
Loggerhead (Laloë *et al.*, 2014)	Cabe Verde	29, 28.8 and 29.2	Mathematical modelling - Fit three different T_{piv} and kept 29 °C
Loggerhead (Zbinden et al. 2007)	Zakynthos	29.3	T_{Piv} previously calculated for same population (Mrosovsky *et al.*, 2002)
Loggerhead (Öz *et al.*, 2004)	Turkey	29	T_{piv} previously calculated for same population (Kaska *et al.*, 1998)
Loggerhead (Hanson *et al.*, 1998)	Florida	29	T_{piv} previously calculated for same population (Mrosovsky, 1988)
Loggerhead (Tanner *et al.*, 2019)	Cabo Verde	29.25	T_{piv} previously calculated for different population (Marcovaldi *et al.*, 1997)
Loggerhead (Jribi *et al.*, 2014)	Tunisia	29.7	T_{piv} previously calculated for different population (Mrosovsky *et al.*, 2002)


A
B
C
1
0
Proportion Male
T_{piv}
T_{piv}
T_{piv}
T_{piv}
Temperature
Temperature
Temperature

Testosterone

Estradiol

Testosterone

Estradiol

Testosterone

Testosterone

Estradiol

Testosterone

Estradiol

Testosterone

Estradiol

= 1

< 1

Estradiol:Testosterone

Maternal Environment

- Sea surface temperatures
- Food availability
- Sea level rise
- Coastal development
- Disturbance by human/predators

Maternal contribution to offspring

Behavioural Plasticity

Maternal nesting behaviours can modify the incubation environment:

- Nest site selection
- Nest depth
- Shift in phenology

Physiological Plasticity

Yolk contents provide priming material for molecular cascades:

- Estradiol
- Testosterone

Genetic Inheritance

Examples:

- SNP at *Cirbp* locus
- Intron retention in *Jarid2* and *Jmjd3* genes

Incubation Environment

- Air temperature
- Precipitation
- Sand albedo
- Humidity

Incubation temperature

Molecular trigger of gene regulation

Molecular Cascades

Female pathway genes:

- *FoxL2*
- *Rspo1*
- *Dax1*
- *Sf1*
- *Wnt4*
- *Cyp19a1*

Male pathway genes:

- *Sox9*
- *AMH*
- *Dmrt1*